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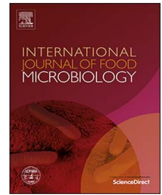
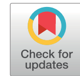


Evaluating the probiotic potential and technological characteristics of yeasts implicated in cv. Kalamata natural black olive fermentation



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A R T I C L E I N F O

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A B S T R A C T

In the present study, 49 yeast strains previously isolated from cv. Kalamata table olive fermentation were assessed for their probiotic potential and technological characteristics. The probiotic assays included the *in vitro* survival in simulated gastric and pancreatic digestions, surface adhesion to the intestinal cell line Caco-2, hydrophobicity, autoaggregation and haemolytic activity. The technological features of the strains were also elucidated in terms of enzymatic activity and susceptibility to diverse salt levels (0–250 g/L) and pH values (3.5, 5.0, and 6.5). The obtained results indicated that during the simulated gastric and pancreatic digestions, 42 out of the 49 yeast strains presented overall survival rate higher than 50%, while 24 strains showed survival percentage higher than 70% at the end of the digestions. Furthermore, the majority of the assayed strains presented hydrophobicity percentage higher than 75%, while the autoaggregation ability ranged between 72 and 91%. None of the strains showed haemolytic activity. The majority of the strains presented high tolerance to salt with some strains exhibiting tolerance even at salt concentrations higher than 200 g/L. Concerning the enzymatic activity, 45 strains presented valine and cystine arylamidase activity, while positive reactions for the enzymes β- and α-glucosidase were observed for 27 and 14 strains, respectively. Moreover, 11 strains presented α-galactosidase and alkaline phosphatase activity. From the total number of studied yeasts, the strain Y34 belonging to *Saccharomyces cerevisiae* presented positive results in the majority of both probiotic and technological assays and thus it could be considered a potential starter either as single or as combined culture with lactic acid bacteria in the fermentation of Greek-style natural black olives.

1. Introduction

Table olive fermentation is driven by the autochthonous microbiota of the olives, namely lactic acid bacteria (LAB) and yeasts, which dominate the process (Sánchez-Gómez et al., 2006). These diverse microbial groups and their metabolic activities determine the sensorial characteristics and the stability of the final product. Brine acidification occurs through the activity of LAB through the production of lactic acid, while the organoleptic profile is affected by the production of specific metabolites and volatile compounds due to yeast activity (Alves et al., 2012). It has been reported recently that in natural black table olive fermentation, yeasts are the dominant species throughout the process, while LAB appear at the end of the fermentation, indicating the importance of these microorganisms in table olive processing (Tufariello et al., 2015). Although specific yeasts may cause spoilage to the olives through the production of CO_2 and undesirable metabolites leading to the deterioration of the quality of the final product (Arroyo-López et al., 2012; Bevilacqua et al., 2015), there are also strains with important probiotic and technological features (Bonatsou et al., 2017). The use of these microorganisms in olive fermentation and the assessment of their beneficial effects *in vivo* may ascribe to table olives the aspect of a functional food that could promote health and well-being. Yeasts have the ability to enhance the aroma and taste of the fermented olives through the metabolic activity of specific enzymes produced by several strains such as lipases and esterases, which increase the free fatty acid content and thus result in the formation of several aromatic

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compounds such as ethanol, glycerol, higher alcohols and other desirable volatile compounds that determine the flavor of the final product (Arroyo-López et al., 2012; Bevilacqua et al., 2012). Furthermore, biodegradation of polyphenols by specific yeast strains through the production of the enzyme β-glucosidase could lead to the debittering of olives without the use of any chemical treatment (Arroyo-López et al., 2008; Bautista-Gallego et al., 2011). In addition, the ability of specific yeast strains to produce enzymes such as phosphatases and phytases is an important technological feature as these enzymes can degrade phytic complexes and release inorganic phosphorous to the cells (Olstorpe et al., 2009).

Natural black table olive fermentation takes place into brine containing 80–100 g/L or even higher NaCl concentration that can be inhibitory for the growth of the microbial consortia involved in fermentation, namely LAB and yeasts. The ability of yeasts to grow at high salt concentrations can be a critical factor for the determination of the strains which will eventually dominate during this process, as well as for the interaction among them (Romero-Gil et al., 2013). Several studies have shown that salt concentrations higher than 80 g/L favored the growth of yeasts, while LAB were either undetectable or maintained at lower populations or detected at the last stage of the process (Bleve et al., 2014, 2015; Tassou et al., 2002; Tufariello et al., 2015). Moreover, yeasts are able to survive and even grow at low pH values and in highly acidified environments (Psomas et al., 2001). Estimation of the non-inhibitory (NIC) and the minimum inhibitory (MIC) concentrations for salt at different pH values could reveal which strains can survive and dominate throughout fermentation (Bevilacqua et al., 2012). Apart from the technological characteristics, yeasts exhibit potential probiotic properties. There are several *in vitro* tests used for the assessment of the probiotic potential of yeast strains, namely, the resistance at low pH values during gastric digestion, the resistance to bile salts and high pH values during pancreatic digestion, the ability to adhere to epithelial cells, the ability to reduce the adherence of pathogens to mucosal surfaces, and the antimicrobial activity against pathogens (Bevilacqua et al., 2012; Bonatsou et al., 2015; Porru et al., 2018).

The present work concerns the multifunctional traits of yeasts isolated from fermented Greek table olives. For this purpose, 49 yeast strains isolated from natural black cv. Kalamata olives, processed with the traditional Greek-style method, were investigated for their technological characteristics (enzymatic activity, resistance to diverse values of salt and pH) and probiotic potential (survival to gastric, pancreatic and overall digestions, autoaggregation, hydrophobicity, adhesion to Caco-2 cells, and haemolytic activity). Multivariate analysis based on hierarchical cluster analysis (HCA) was applied to the dataset derived from the aforementioned assays to discriminate yeasts into clusters according to their probiotic and technological features and eventually select the most promising strains to be used as starter cultures either alone or in co-culture with LAB during the Greek-style processing of table olives.

2. Materials and methods

2.1. Yeast strains

A total of 49 yeast strains were investigated in this study as indicated in Table 1. They have been previously isolated from the spontaneous fermentation of Greek natural black cv. Kalamata olives (Bonatsou et al., 2018) and identified at the strain level by rep-PCR using the primer $(GTG)_5$ in combination with sequence analysis of the D1/D2 domain of the 26S gene and blast analysis. All strains were deposited in the Culture Collection of the Laboratory of Microbiology and Biotechnology of Foods of the Agricultural University of Athens.

2.2. Tolerance of yeasts to salt at different pH values

The experiment was performed as previously described (Bonatsou et al., 2015) with slight modifications. One single colony of each isolate was inoculated into 5 mL of Yeast Mold (YM) broth medium (Yeast extract 3.0 g/L, Malt extract 3.0 g/L, Dextrose 10.0 g/L, Bacteriological peptone 5.0 g/L). After 48 h of incubation at 28 °C, an appropriate volume of the culture was centrifuged at 5000 ×*g* for 10 min, washed with sterile ringer solution and re-suspended in 5 mL of a sterile saline solution to obtain a final concentration of about 6.5 $\log_{10}$ CFU/mL, which was confirmed by plating on YM agar. These yeast suspensions were used in further trials to determine the tolerance of yeasts to salt at different pH values. Sterilized YM broth was supplemented with NaCl to obtain the following final concentrations of salt in the media: 0, 30, 50, 70, 90, 110, 140, 180, 200, and 250 g/L, at three different pH values namely, 3.5, 5.0, and 6.5. All trials were carried out in triplicate generating a total of 4410 growth curves (10 NaCl levels × 49 yeasts × 3 pH values × triplicate). Growth was monitored by means of optical density (OD) measurements in an automated spectrophotometer (Biotek synergy, Winooski, Vermont, USA) at 420 nm. Measurements of OD were recorded every 12 h for a period of 7 days. The wells were filled with 330 μL of medium and inoculated with 20 μL of yeast inoculum. NIC (non-inhibitory concentration) and MIC (minimum-inhibitory concentration) values of the yeast strains for different NaCl/pH combinations were calculated by comparing the area under the OD/time curve of the control (absence of salt) with the areas of the trials (presence of salt at different pH values). Growth was indicated by a reduction in the area under the OD/time curve relative to the control. The amount of growth for each NaCl/pH condition, denoted as the fractional area (*fa*), was estimated using the ratios of the test area ($area_{test}$) to that of the control of the yeast ($area_{cont}$). The graphical representation of the values of *fa versus* the natural logarithm (ln) of salt concentration at each different pH value produced a sigmoid curve that was fitted with the modified Gompertz equation to calculate the MIC and NIC values as detailed elsewhere (Lambert and Pearson, 2000).

Table 1
The 49 yeast strains investigated in the present study.

Yeast strain	Number of isolates	Code
Rhodotorula glutinis	3	Y1, Y2, Y3
Citeromyces matrinensis	1	Y4
Pichia kluyveri	2	Y5, Y6
Cystofilobasidium bisporidii	1	Y7
Candida naeodendra	2	Y8, Y9
Candida diddensiae	3	Y10, Y11, Y12
Metschnikowia pulcherrima	3	Y13, Y14, Y15
Rhodotorula mucilaginosa	1	Y16
Pichia manshurica	3	Y17, Y18, Y19
Pichia guilliermondii	2	Y21, Y22
Zygoascus hellenicus	3	Y23, Y24, Y25
Candida boidinii	6	Y26, Y27, Y28, Y29, Y30, Y31
Saccharomyces cerevisiae	6	Y32, Y33, Y34, Y35, Y36, Y37
Aureobasidium pullulans	6	Y38, Y39, Y40, Y41, Y42, Y43
Candida molendinolei	7	Y44, Y44, Y45, Y46, Y47, Y48, Y49, Y50

2.3. Enzymatic assay

For the estimation of the enzymatic activity of the isolated strains, the API-ZYM kit (Bio-Merieux, France) was used following the instructions of the manufacturer. The specific kit is designed for the semi-quantification of 19 different enzymatic activities namely, alkaline phosphatase, esterase, esterase lipase, lipase, lysine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, Naphthol-AS-BI- phosphohydrolase (NAGase), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, and α-fucosidase. After incubation for 2 days at 28 °C, the yeast cultures were centrifuged, the supernatant was removed and the pellets were re-suspended in an isotonic buffer (0.85% w/v NaCl).

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